

COMMUNICATION

First Synthesis of the Antiangiogenic Homoisoflavanone, Cremastranone

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An antiangiogenic homoisoflavanone, cremastranone, was synthesized for the first time. This scalable synthesis, which includes selective demethylation, could be used to develop lead molecules to treat angiogenesis-induced eye diseases. Synthetic cremastranone inhibited the proliferation, migration and tube formation ability of human retinal microvascular endothelial cells, important steps in pathological angiogenesis.

Retinopathy of prematurity (ROP), proliferative diabetic retinopathy (DR) and neovascular ("wet") age-related macular degeneration (wet AMD) are three major ocular diseases, affecting children, adults and elderly people.¹ These diseases are characterized by abnormal formation of blood vessels in the eye through the process of pathological angiogenesis. The blood vessels formed during pathological angiogenesis in the eye are fragile, porous and not completely differentiated. As a result haemorrhage, retinal detachment, fibrotic scarring and rapid photoreceptor degeneration occurs in the eye leading to vision loss.

Nearly 2 million people are affected by wet AMD in the United States, while about 75% of diabetic patients show clinical symptoms of DR.¹ Blocking pathological angiogenesis in the eyes is the current treatment strategy for these diseases. The current mainstay of therapy is the use of anti-VEGF biologics. However, these treatments are associated with vision damaging side effects, unfavourable cost to benefit ratio, and incomplete patient response.²⁻⁵ Hence there is a critical requirement for new therapies. Towards this goal, we are pursuing development of small molecules as drug leads for these diseases, based on natural products.

Homoisoflavanones are a small class of naturally occurring oxygen heterocycles that have been isolated from many geographically diverse plant genera. These compounds are categorized into four types: 3-benzyl-4-chromanones, 3-benzylidene-4-chromanones, 3-benzyl-3-hydroxy-4-chromanones and scillascillins.⁶ In general, these compounds are reported to show anti-

inflammatory, anti-oxidative, anti-bacterial, anti-cancer, anti-histaminic, anti-viral, and anti-phosphodiesterase activities. Cremastranone (**1**), 5,7-dihydroxy-3-(3-hydroxy-4-methoxybenzyl)-6-methoxychroman-4-one, is a 3-benzyl-4-chromanone that has been isolated from the plants *Muscari armeniacum*,⁷ *Chionodoxa luciliae*,⁸ *Scilla natalensis*,⁹ *Merwillia plumbea*,¹⁰ and *Cremastra appendiculata* (D. Don)¹¹ (**1**; Fig. 1). The bulb of the orchid *C. appendiculata* is used in East Asia as a traditional medicine, taken internally to treat several cancers, and applied externally for skin lesions.

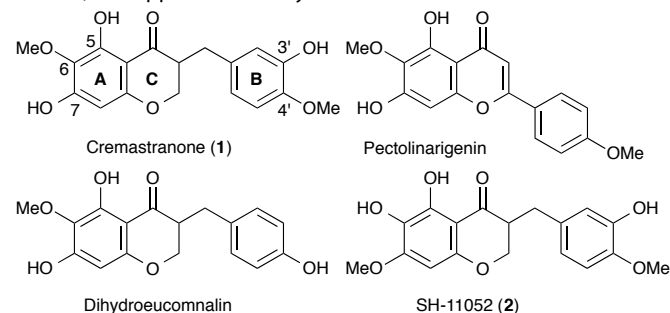


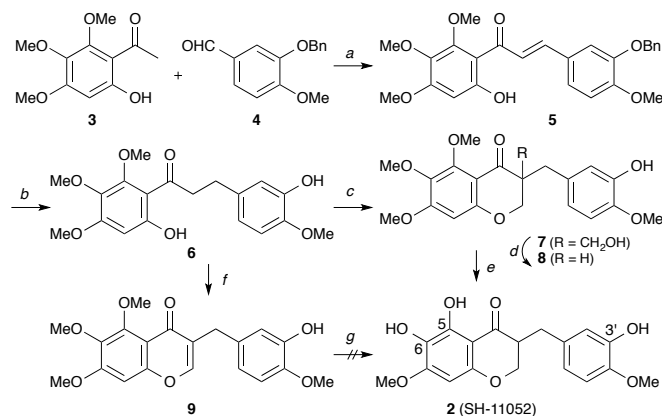
Fig. 1 The structures of cremastranone (**1**) and its congeners.

Cremastranone (**1**) was previously reported to possess anti-angiogenic activity both *in vitro* and *in vivo* and was identified as a potent inhibitor of the proliferation of human umbilical vein endothelial cells (HUVECs).¹¹ Also **1** inhibited vascular tube formation and new vessel growth induced by basic fibroblast growth factor. Its anti-angiogenic properties were further confirmed *in vivo* in the laser-induced choroidal neovascularization and oxygen induced retinopathy mouse models,^{12, 13} used for testing novel therapies for wet AMD and ROP, respectively. Moreover, injection of compound **1** into the vitreous of normal adult mice showed no short-term cytotoxic or inflammatory effects on the retina, nor did it induce apoptosis of retinal cells.¹³ These results suggest that proliferative ocular vascular diseases such as ROP, DR, and AMD may be targeted using compound **1** or its derivatives. Other recent work has

suggested that **1** has potent anti-inflammatory activity in the context of UV-induced skin inflammation¹⁴ and allergy.¹⁵

So far, the syntheses of 5,7-dihydroxy-6-methoxyflavones and 5,7-dihydroxy-6-methoxyhomoisoflavanones have been reported (Fig. 1).^{16, 17} But in spite of cremastranone's interesting biological activities and the synthesis of these congeners, to the best of our knowledge, synthesis of **1** has not yet been reported. To allow future profiling and development of this homoisoflavanone and its analogs, we sought to develop a scalable synthesis suitable for quickly securing multi-gram quantities. During our previous synthetic approach toward cremastranone, the regioisomer (SH-11052, **2**) was generated due to the challenge of selective deprotection of methyl phenyl ethers.¹⁸ Herein, we describe a short and efficient synthesis of cremastranone (**1**) featuring a solution to the issue of selective deprotection of the methyl phenyl ether. Moreover, we show for the first time that synthetic cremastranone has biological properties consistent with the natural-source compound, in a disease relevant cell model.

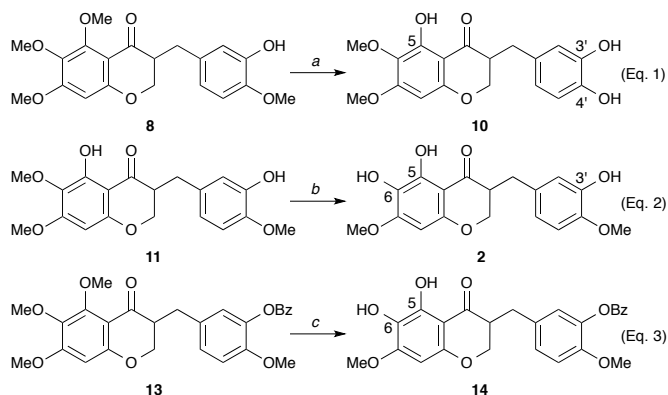
Our plan for the preparation of cremastranone entailed 4-chromanone formation and deprotection of a methyl aryl ether.¹⁹ This strategy would permit significant flexibility in the synthesis of **1** and thereby establish a platform that leads to a variety of derivatives for further structure-activity relationship studies. In addition, we envisioned our synthesis would become very useful for the syntheses of similar interesting homoisoflavanones.



Scheme 1 The initial synthetic approach to cremastranone (**1**). Reagents and conditions: a) KOH, MeOH, 0°C, 54%; b) Pd/C, HCO₂Na, HCO₂H, 60°C, 79%; c) formalin, NaOH, 60°C; d) K₂CO₃, EtOH, 47% for 2 steps; e) TMSI (4 eq.), CHCl₃, 50°C, 49%; f) PCl₅, BF₃·OEt₂, DMF, 62%; g) HBr, AcOH then H₂, Pd/C, MeOH.

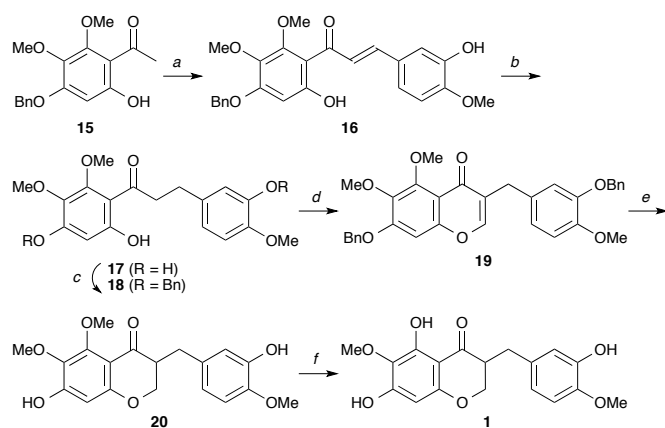
As shown in Scheme 1 and described previously,¹⁸ our initial approach toward the synthesis of cremastranone (**1**) began with an aldol condensation of the 6'-hydroxy-2',3',4'-trimethoxyacetophenone **3** with 3-benzyloxy-4-methoxybenzaldehyde **4**. Catalytic hydrogenation of the chalcone **5** and the cyclization of the dihydrochalcone **6** with formaldehyde led to the desired 4-chromanone **8** after byproducts (e.g. **7**) were treated with K₂CO₃. Finally, in order to remove two methyl groups selectively, the 4-chromanone **8** would be demethylated with a variety of reagents. However, the selective removal of two methyl groups under most conditions was not satisfactory. Among them, the demethylation using an excess of TMSI in CHCl₃ gave the best conversion,²⁰ but its

product was not identical to cremastranone but rather its regioisomer, called SH-11052 (**2**). Its structure was elucidated by 2D-NMR spectroscopies such as NOESY and HMBC.¹⁸ To overcome the unwanted result, the alternative 4-chromanone **9** cyclized by DMF, PCl₅ was demethylated and hydrogenated.²¹ Similarly, the desired product was not afforded from **9**. Surprisingly, treatment of **8** with BBr₃ led to the other isomer demethylated on the C4' position (**10**).²² (Eq. 1, Scheme 2) The next trial was to remove the intermediates which were demethylated only on the C5 position or protected with a benzoyl group on the C3' position. (Eq. 2 and 3, Scheme 2) Unfortunately, the free OH group at the C7 position could not be generated from any intermediates bearing 5,6,7-trimethoxy-4-chromanone.



Scheme 2 The study of the demethylation of 5,6,7-trimethoxy-4-chromanone. Reagents and conditions: a) BBr₃ (or BCl₃) (2.5 eq.), CH₂Cl₂, -78°C, 65%; b) TMSI, CHCl₃, reflux, 38%; c) TMSI (4 eq.), CHCl₃, reflux, 52%.

Finally, our key solution for an alternative route to cremastranone (**1**) was the generation of the phenol group on the C7 position prior to the C6 and C4' positions. Thus 4'-benzyloxy-6'-hydroxy-2',3'-dimethoxyacetophenone **15** was chosen as the starting material instead of **3** (Scheme 3). As reported before, **15** was prepared from 2',4',6'-trihydroxyacetophenone with several steps.²³ Aldol condensation of **15** with isovanillin, followed by catalytic hydrogenation of the chalcone **16** under H₂ and Pd/C afforded the dihydrochalcone **17**. With the dihydrochalcone **17** in hand, the formation of the 4-chromanone was directly attempted using *N,N*-dimethylformamide dimethyl acetal. However, the methylation of a phenol group accompanied this reaction. To overcome the side reaction, the dihydrochalcone **17** was carefully treated with benzyl bromide to afford the bis(benzyl ether) **18** which was converted to the corresponding 4-chromanone **19**. To this end, catalytic hydrogenation of **19** led to the desired 4-chromanone **20**. Finally, methyl groups were removed by 2 equivalents of TMSI to give cremastranone (**1**). Thus the antiangiogenic homoisoflavanone cremastranone is available in 6 steps and 26.8% overall yield from the acetophenone **15**. Although our synthetic cremastranone is a racemate, its chemical shifts in ¹H- and ¹³C-NMR spectra match those of the reported natural product (Supplementary Table 1).⁷ To date, the configuration at C3 for natural-source compound **1** has not been reported. Further experiments are necessary to determine the absolute configuration of the C3 position in compound **1**.



Scheme 3 First synthesis of (±)-cremastranone (**1**). Reagents and conditions: a) isovanillin, KOH, EtOH, rt, 53%; b) H₂, Pd/C, MeOH, 94%; c) K₂CO₃, benzyl bromide, acetone, reflux, 89%; d) (CH₃)₂NCH(OCH₃)₂, toluene, reflux, 80%; e) H₂, Pd/C, MeOH, 87%; f) TMSI (2 eq.), CHCl₃, 60°C, 87%.

Previously, cremastranone isolated from *C. appendiculata* was tested for its anti-proliferative activity against an endothelial cell model, human umbilical vein endothelial cells (HUVECs), and the 50% growth inhibitory concentration (GI₅₀) value was reported to be 1.5 μM.¹¹ Hence, we tested the anti-proliferative activity of synthetic cremastranone **1** on HUVECs in an alamarBlue fluorescence cell proliferation assay and the GI₅₀ was observed to be 377 nM. The slightly higher potency of synthetic **1** might be attributed to a difference in the proliferation assays used or compound purity. Further, we tested the anti-proliferative activity of **1** against a more ocular disease relevant endothelial cell model, human retinal microvascular endothelial cells (HRECs). We observed clear dose dependent inhibition of HREC proliferation by **1** in complete medium with a GI₅₀ of 217 nM (Fig. 2a). In addition we also tested the proliferation of HRECs activated by vascular endothelial growth factor, a potent inducer of angiogenesis, in the presence of **1** and found the GI₅₀ to be comparable (276 nM). Previously we reported a GI₅₀ of 43 μM for regioisomer SH-11052 (**2**),¹⁸ which differed only in the positions of hydroxyl and methoxy groups at C6 and C7 as compared to **1**. The importance of positions of the groups on the 'A' ring suggest that a structure-activity relationship can be established.

We then measured the endothelial cell specificity of **1** by measuring its effects on the proliferation of non-endothelial ocular cell lines, Y79 (retinoblastoma cell line), 92-1 (uveal melanoma cell line) and ARPE-19 (retinal pigmented epithelial cells). The anti-proliferative potency of **1** on these non-endothelial cell lines was significantly lower than that on endothelial cells with GI₅₀ values of 9.8 μM, 47 μM, and >250 μM on Y79, 92-1, and ARPE-19 cells respectively, indicating that compound **1** has highly specific antiproliferative activity toward endothelial cells. We further confirmed the inhibition of HREC proliferation by **1** in a secondary assay by monitoring the incorporation of thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU) into DNA of endothelial cells in the presence of different concentrations of **1**. In this EdU incorporation assay, compound **1** inhibited the DNA synthesis of HRECs in a dose dependent manner (Fig. 2b, e).

After establishing the inhibition of HREC proliferation by **1**, we tested its anti-angiogenic activity *in vitro*. We tested the ability of

HRECs to form tubes *in vitro* in a Matrigel tube formation assay, which recapitulates *in vitro* major events of physiological angiogenesis. Cremastranone **1** prevented the formation of closed tube structures in a dose dependent manner as measured by both tube length (Fig. 2c, f) and number of polygons formed (data not shown). This is consistent with previous findings with the natural-source cremastranone in HUVECs.¹¹ Migration is also an important step in the angiogenesis process, wherein endothelial cells move from pre-existing capillaries to the site of blood vessel formation. We measured this ability of HRECs to migrate using the standard scratch-wound assay, in which HRECs were allowed to grow to confluency, then a scratch was introduced and movement of endothelial cells into the scratched area from the surrounding population was measured in the presence of various concentrations of **1** (Fig. 2d). The migration of HRECs was inhibited by **1** in a dose dependent manner.

In summary, a novel scalable strategy to synthesize the biologically active homoisoflavanone cremastranone was developed, and the resulting compound showed potent activity in cell models. Together, these results confirm the anti-angiogenic activity of synthetic cremastranone **1** in a disease-relevant cell type, and pave the way for development of analogues with higher potency and better pharmacological properties to treat blinding ocular diseases caused by pathological angiogenesis.

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Notes

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Electronic Supplementary Information (ESI) available: synthetic methodology, compound characterization, and biological methods. See DOI: 10.1039/c000000x/

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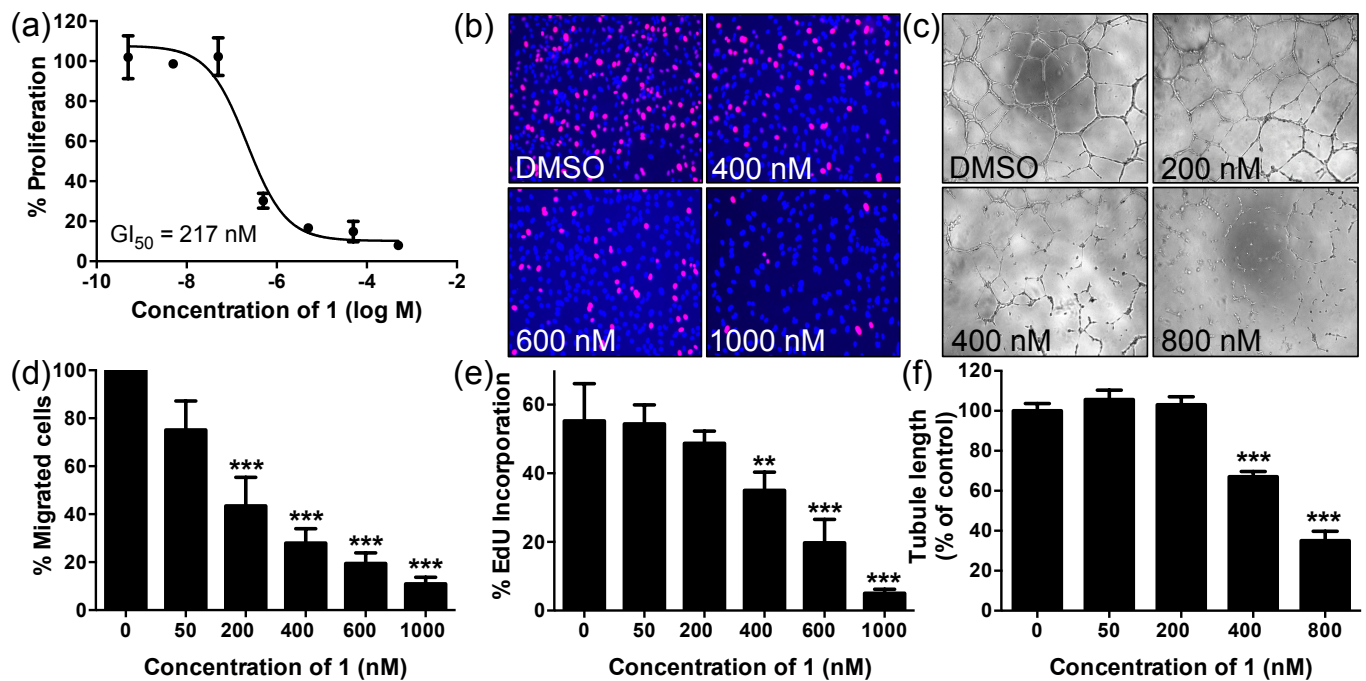


Fig. 2 Synthetic cremastranone (**1**) blocks in vitro angiogenesis of human retinal microvascular endothelial cells (HRECs) in a dose-dependent manner. (a) The effect of **1** on proliferation of HRECs was tested using an alamarBlue fluorescence assay. (b, e) In an EdU incorporation assay, **1** blocked DNA synthesis as evidenced by a decrease in incorporation of EdU (pink) into the nuclei of HRECs (nuclei, blue). (c, f) Tube formation by HRECs on Matrigel was blocked by **1**; the extent of tube formation was measured as tubule length. (d) Migration of HRECs in a scratch-wound assay was inhibited by **1**. Original magnifications: 40x. The data points in all graphs indicate mean \pm SEM; ** represents $P < 0.01$; *** represents $P < 0.001$ (ANOVA with Dunnett's post hoc tests). Each of the panels is representative of three independent experiments.

Electronic supporting information for

First Synthesis of the Antiangiogenic Homoisoflavanone, Cremastranone

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Synthetic Methodology

All starting materials and reagents were obtained commercially and were used without further purification. Tetrahydrofuran was distilled from sodium benzophenone ketyl. Dichloromethane and acetonitrile were freshly distilled from calcium hydride. All solvents used for routine product isolation and chromatography were of reagent grade and glass distilled. Reaction glassware was dried at 100 °C before use, and air- and moisture-sensitive reactions were performed under argon. Flash column chromatography was performed using silica gel 60 (230–400 mesh, Merck) with the indicated solvents. Thin-layer chromatography was performed using 0.25 mm silica gel plates (Merck). Mass spectra were obtained using a Waters Auto Purification instrument. ¹H and ¹³C spectra were recorded on either a Bruker 600MHz spectrometer or a Bruker 400MHz spectrometer as solutions in deuteriochloroform (CDCl₃) or methanol-d₄ (CD₃OD). ¹H NMR data are reported in the order of chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet and/or multiple resonances), number of protons, and coupling constant (*J*) in Hertz (Hz).

5,6-dihydroxy-3-(3'-hydroxy-4'-methoxybenzyl)-7-methoxychroman-4-one (2)

SH-11052 (2) and intermediates 5, 6, 7, and 8 were synthesized as previously described.¹

3-(3'-hydroxy-4'-methoxybenzyl)-5,6,7-trimethoxy-4H-chromen-4-one (9)

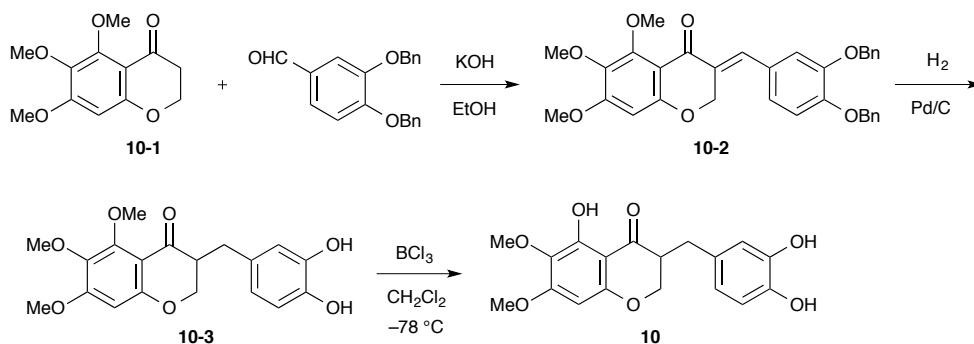
A solution of PCl_5 (180 mg, 0.86 mmol) in DMF (2.5 mL) was stirred at 20 °C for 20 min. To the reaction mixture was added $\text{BF}_3\cdot\text{Et}_2\text{O}$ (0.22 mL, 1.73 mmol) and the dihydrochalcone 1-(6-hydroxy-2,3,4-trimethoxyphenyl)-3-(3'-hydroxy-4'-methoxyphenyl)propan-1-one (**6**) (200 mg, 0.55 mmol) at 20 °C, then the mixture was stirred for 4 h followed by the addition of 1N HCl (2 mL) and dilution with ethyl acetate. The organic layer was washed with water and brine, dried over MgSO_4 , and concentrated in vacuo. The residue was purified by silica gel column chromatography (ethyl acetate / *n*-hexane = 1 : 4) to afford the chromone (**9**) (128 mg, 62%). ^1H -NMR (400 MHz, CDCl_3) δ 7.34 (s, 1H), 6.83-6.79 (m, 2H), 6.74-6.73 (m, 1H), 6.59 (s, 1H), 5.49 (s, 1H), 3.94 (s, 3H), 3.90 (s, 3H), 3.86 (s, 3H), 3.67 (s, 2H); ^{13}C -NMR (100 MHz, CDCl_3) δ 175.9, 157.5, 154.7, 152.6, 151.0, 146.5, 144.1, 140.2, 130.6, 125.2, 121.6, 114.3, 112.9, 111.7, 95.9, 62.0, 61.4, 56.2, 55.9, 31.1.; HRMS (ESI): mass calcd for $\text{C}_{20}\text{H}_{20}\text{O}_7$ $[\text{M} + \text{H}]^+$, 373.1287; found, 373.1280.

3-(3',4'-dihydroxybenzyl)-5-hydroxy-6,7-dimethoxychroman-4-one (10)

i) For synthesis from compound (8),

To a CH_2Cl_2 (3 mL) solution of the 3-(3-hydroxy-4-methoxybenzyl)-5,6,7-trimethoxychroman-4-one (**8**) (42 mg, 0.11 mmol), boron tribromide (1.0 M solution of CH_2Cl_2 , 280 μL , 0.28 mmol) was added at -78 °C. After stirring for 1 h, the reaction mixture was heated to ambient temperature, quenched with methanol, and stirred for 30 min. The organic phase was washed with water and brine, dried over MgSO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography (ethyl acetate / *n*-hexane = 1 : 2) to afford demethylated compound (**10**) (25 mg, 65%); ^1H -NMR (600 MHz, CD_3OD) δ 6.71 (d, 1H, J = 6.8 Hz), 6.67 (d, 1H, J = 2.4 Hz), 6.55 (dd, 1H, J = 7.8 and 1.8 Hz), 6.14 (s, 1H), 4.29 (dd, 1H, J = 11.4 and 4.2 Hz), 4.13 (dd, 1H, J = 11.4 and 7.8 Hz), 3.87 (s, 3H), 3.73 (s, 3H), 3.03 (dd, 1H, J = 13.8 and 4.8 Hz), 2.82 (m, 1H), 2.59 (dd, 1H, J = 14 and 10 Hz); ^{13}C -NMR (150 MHz, CD_3OD) δ 200.6, 162.3, 160.6, 156.2, 146.5, 145.2, 131.3, 130.8, 121.5, 117.1, 116.5, 103.6, 92.5, 70.5, 61.1, 56.8, 33.1.

ii) For synthesis from 5,6,7-methoxy-4-chromanone (**10-1**),



To a solution of 5,6,7-methoxy-4-chromanone (**10-1**) (108 mg, 0.42 mmol) in benzene (5 mL) was added 3',4'-bis(benzyloxy)benzaldehyde (102 mg, 0.45 mmol) and PTSA (9 mg, 0.04 mmol) at 0 °C. The reaction mixture was refluxed for 12 h. After cooling to ambient temperature, the reaction mixture was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (ethyl acetate / *n*-hexane = 1 : 2) to afford (*E*)-3-(3',4'-bis(benzyloxy)benzylidene)-5,6,7-trimethoxychroman-4-one (**10-2**) (117 mg, 51%). And then to an anhydrous MeOH solution of the benzylidene (**10-2**) (78 mg, 0.15 mmol) and 10% Pd/C (8 mg) was placed under an atmosphere of hydrogen. After stirring for 1 h, the reaction mixture was diluted with ethyl acetate, filtered through a Celite pad and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate / *n*-hexane = 1 : 5) to afford the homoisoflavanone (**10-3**) (42 mg, 77%). ¹H-NMR (600 MHz, CDCl₃) δ 6.78 (d, 1H, *J* = 4.8 Hz), 6.76 (s, 1H), 6.57 (dd, 1H, *J* = 7.8 and 2.4 Hz), 6.22 (s, 1H), 4.24 (dd, 1H, *J* = 11 and 4.2 Hz), 4.09 (dd, 1H, *J* = 12 and 7.2 Hz), 3.90 (s, 3H), 3.84 (s, 3H), 3.77 (s, 3H), 3.05 (dd, 1H, *J* = 7.8 and 4.8 Hz), 2.68 (m, 1H), 2.58 (dd, 1H, *J* = 14 and 10 Hz); ¹³C-NMR (150 MHz, CDCl₃) δ 192.7, 160.0, 159.7, 154.3, 144.1, 142.8, 137.3, 130.5, 121.3, 115.9, 115.3, 108.3, 96.1, 69.0, 61.6, 61.3, 56.1, 48.5, 32.5; HRMS (ESI): mass calcd for C₁₉H₂₀O₇ [M + H]⁺, 361.1287; found, 361.1275. To a CH₂Cl₂ (3 mL) solution of the 3-(3',4'-dihydroxybenzyl)-5,6,7-trimethoxychroman-4-one (**10-3**) (38 mg, 0.1 mmol) boron trichloride (1.0 M solution of CH₂Cl₂, 500 μL, 0.5 mmol) was added at -78 °C. After stirring for 1 h, the reaction mixture was heated to ambient temperature, quenched with methanol, and stirred for 30 min. The organic phase was washed with water and brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography (ethyl acetate / *n*-hexane = 1 : 2) to afford demethylated compound (**10**) (13 mg, 38%). ¹H-NMR (600 MHz, CD₃OD) δ 6.71 (d, 1H, *J* = 6.8 Hz), 6.67 (d, 1H, *J* = 2.4 Hz), 6.55 (dd, 1H, *J* = 7.8 and 1.8 Hz), 6.14 (s, 1H), 4.29 (dd, 1H, *J* = 11.4 and 4.2 Hz), 4.13 (dd, 1H, *J* = 11.4 and 7.8 Hz), 3.87 (s, 3H), 3.73 (s, 3H), 3.03 (dd, 1H, *J* = 13.8 and 4.8 Hz), 2.82 (m, 1H), 2.59 (dd, 1H, *J* = 14.4 and 10.2 Hz); ¹³C-NMR (150 MHz, CD₃OD) δ 200.6, 162.3, 160.6, 156.2, 146.5, 145.2, 131.3, 130.8, 121.5, 117.1, 116.5, 103.6, 92.5, 70.5, 61.1, 56.8, 33.1; HRMS (ESI): mass calcd for C₁₈H₁₈O₇ [M + H]⁺, 347.1131; found, 347.1122

5-hydroxy-3-(3'-hydroxy-4'-methoxybenzyl)-6,7-dimethoxychroman-4-one (11)

To a solution of 3-(3'-hydroxy-4'-methoxybenzyl)-5,6,7-trimethoxychroman-4-one (**8**) (70 mg, 0.187 mmol) in CHCl₃ (2 mL) was added TMSI (53 μ L, 0.374 mmol) at 0 °C and the reaction mixture was heated at 60 °C for 1 h. The mixture was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (ethyl acetate / *n*-hexane = 1 : 2) to afford compound **11** (49 mg, 73%). ¹H-NMR (600 MHz, CD₃OD) δ 6.85 (d, 1H, *J* = 7.8 Hz), 6.71 (d, 1H, *J* = 1.8 Hz), 6.66 (dd, 1H, *J* = 8.4 and 2.4 Hz), 6.14 (s, 1H), 4.29 (dd, 1H, *J* = 11.4 and 4.2 Hz), 4.12 (dd, 1H, *J* = 11.4 and 7.2 Hz), 3.86 (s, 3H), 3.83 (s, 3H), 3.73 (s, 3H), 3.09 (dd, 1H, *J* = 13.8 and 4.2 Hz), 2.87-2.82 (m, 1H), 2.64 (dd, 1H, *J* = 14.4 and 10.2 Hz); ¹³C-NMR (150 MHz, CD₃OD) δ 200.5, 162.4, 160.7, 156.2, 148.0, 147.8, 132.3, 131.4, 121.4, 117.1, 113.0, 103.7, 92.6, 70.6, 61.2, 56.8, 56.5, 48.2, 33.1; HRMS (ESI): mass calcd for C₁₉H₂₀O₇ [M + H]⁺, 361.1287; found, 361.1277.

2'-methoxy-5'-((5,6,7-trimethoxy-4-oxochroman-3-yl)methyl)phenyl benzoate (13)

To a solution of 3-(3'-hydroxy-4'-methoxybenzyl)-5,6,7-trimethoxychroman-4-one (**8**) (149 mg, 0.39 mmol) in CH₂Cl₂ (2.5 mL) was added Et₃N (0.11 mL, 0.78 mmol), DMAP (10 mg, 0.08 mmol), and benzoyl chloride (0.05 mL, 0.47 mmol) at 0 °C and the reaction mixture was stirred at ambient temperature for 2 h. The reaction mixture was diluted with ethyl acetate and the organic phase was washed with water and brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate / *n*-hexane = 1 : 2) to afford the benzoate compound (**13**) (182 mg, 80%). ¹H-NMR (400 MHz, CDCl₃) δ 8.18-8.16 (m, 2H), 7.60-7.56 (m, 1H), 7.48-7.44 (m, 2H), 7.06 (d, 1H, *J* = 7.8 Hz), 6.86 (d, 1H, *J* = 1.9 Hz), 6.83 (dd, 1H, *J* = 8.3 and 1.9 Hz), 6.23 (s, 1H), 4.31 (dd, 1H, *J* = 11 and 3.9 Hz), 4.13 (dd, 1H, *J* = 11 and 7.8 Hz), 3.91 (s, 3H), 3.84 (s, 3H), 3.78 (s, 3H), 3.77 (s, 3H), 3.26 (dd, 1H, *J* = 14 and 3.9 Hz), 2.81 (m, 1H), 2.70 (dd, 1H, *J* = 14 and 11 Hz); ¹³C-NMR (100 MHz, CDCl₃) δ 190.9, 164.6, 159.6, 159.2, 154.3, 151.2, 138.5, 137.4, 133.3, 130.1, 129.9, 129.2, 128.3, 128.2, 122.7, 121.2, 113.1, 108.5, 95.9, 68.9, 61.5, 61.1, 56.0, 55.8, 48.2, 32.6.

5'-((5,6-dihydroxy-7-methoxy-4-oxochroman-3-yl)methyl)-2'-methoxyphenyl benzoate (14)

To a solution of benzoate (**13**) (65 mg, 0.136 mmol) in CHCl₃ (1 mL) was added TMSI (77 μ L, 0.54 mmol) at 0 °C and the reaction mixture was heated at 60 °C for 1 h. The mixture was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (ethyl acetate / *n*-hexane = 2 : 3) to afford the compound **14** (32 mg, 52%). ¹H-NMR (400 MHz, CDCl₃) δ 8.20 (m, 2H), 7.63 (m, 1H), 7.51 (m, 2H), 7.09 (d, 1H, *J* = 7.8 Hz), 6.87 (d, 1H, *J* = 2.0 Hz), 6.85 (dd, 1H, *J* = 7.8 and 2.0 Hz),

6.06 (s, 1H), 4.32 (dd, 1H, J = 11 and 3.9 Hz), 4.16 (dd, 1H, J = 11 and 7.3 Hz), 3.90 (s, 3H), 3.82 (s, 3H), 3.26 (dd, 1H, J = 14 and 3.9 Hz), 2.89-2.87 (m, 1H), 2.78 (dd, 1H, J = 14 and 11 Hz); ^{13}C -NMR (100 MHz, CDCl_3) δ 198.4, 164.7, 155.9, 154.7, 151.4, 148.1, 138.8, 136.8, 133.4, 130.2, 129.3, 128.5, 127.3, 123.0, 121.3, 113.2, 102.4, 91.0, 69.2, 56.3, 55.9, 46.8, 32.7, 30.9.

(*E*)-1-(4-(benzyloxy)-6-hydroxy-2,3-dimethoxyphenyl)-3-(3'-hydroxy-4'-methoxyphenyl)prop-2-en-1-one (16)

To a solution of 4'-benzyloxy-6'-hydroxy-2',3'-dimethoxyacetophenone (**15**) (104 mg, 0.34 mmol) in EtOH (6 mL) was added KOH (95 mg, 1.7 mmol) and isovanillin (62 mg, 0.41 mmol) at rt. The reaction mixture was stirred for 48 h at rt. The mixture was concentrated in vacuo. The residue was washed with 2 N HCl solution and brine. Drying over MgSO_4 and removal of the solvent followed by column chromatography on silica gel using (ethyl acetate / *n*-hexane = 1 : 2) gave the chalcone (**16**) (67 mg, 53%) as a yellow solid. ^1H -NMR (600 MHz, CDCl_3) δ 13.68 (s, 1H), 7.81 (d, 1H, J = 15.6 Hz), 7.75 (d, 1H, J = 15.6 Hz), 7.42 (d, 2H, J = 6.6 Hz), 7.38 (t, 2H, J = 7.8 Hz), 7.33 (t, 1H, J = 7.2 Hz), 7.26 (d, 1H, J = 2.4 Hz), 7.11 (dd, 1H, J = 8.4 and 2.4 Hz), 6.85 (d, 1H, J = 8.4 Hz), 6.32 (s, 1H), 5.12 (s, 2H), 3.92 (s, 3H), 3.91 (s, 3H), 3.83 (s, 3H); ^{13}C -NMR (150 MHz, CDCl_3) δ 192.8, 162.4, 159.1, 155.1, 148.7, 145.9, 143.5, 135.8, 135.5, 129.0, 128.7, 128.2, 127.3, 124.6, 122.8, 113.0, 110.5, 109.0, 97.7, 70.6, 61.9, 61.3, 56.0; HRMS (ESI): mass calcd for $\text{C}_{25}\text{H}_{24}\text{O}_7$ $[\text{M} + \text{H}]^+$, 437.1600; found, 437.1620.

1-(4,6-dihydroxy-2,3-dimethoxyphenyl)-3-(3'-hydroxy-4'-methoxyphenyl)propan-1-one (17)

A solution of the chalcone (**16**) (40 mg, 0.11 mmol) and 10% Pd/C (20 mg) in anhydrous MeOH was placed under an atmosphere of hydrogen. After stirring for 1 h, the reaction mixture was diluted with ethyl acetate, filtered through a Celite pad and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate : *n*-hexane = 1 : 2) to afford the dihydrochalcone (**17**) (30 mg, 94%). ^1H -NMR (600 MHz, CDCl_3) δ 13.23 (s, 1H), 6.81 (d, 1H, J = 2.4 Hz), 6.77 (d, 1H, J = 7.8 Hz), 6.71 (dd, 1H, J = 8.4 and 2.4 Hz), 6.27 (s, 1H), 5.60 (s, 1H), 3.90 (s, 3H), 3.85 (s, 3H), 3.80 (s, 3H), 3.3 (t, 2H, J = 7.8 Hz), 2.92 (t, 2H, J = 7.8 Hz); ^{13}C -NMR (150 MHz, CDCl_3) δ 207.6, 161.8, 156.2, 154.4, 145.5, 144.8, 134.7, 132.7, 119.8, 114.56, 110.7, 108.5, 99.1, 60.9, 60.6, 56.0, 44.9, 29.8; HRMS (ESI): mass calcd for $\text{C}_{18}\text{H}_{20}\text{O}_7$ $[\text{M} + \text{H}]^+$, 349.1287; found, 349.1272.

3-(3'-(benzyloxy)-4'-methoxyphenyl)-1-(4-(benzyloxy)-6-hydroxy-2,3 dimethoxyphenyl)propan-1-one (18)

To an acetone (5 mL) solution of the dihydrochalcone **17** (250 mg, 0.72 mmol) were added benzyl bromide (270 mg, 1.6 mmol) and K₂CO₃ (300 mg, 2.2 mmol). After refluxing for 3 h, the reaction mixture was diluted with ethyl acetate and the organic phase was washed with water and brine, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate / *n*-hexane = 1 : 2) to afford the dibenzylated compound (**18**) (340 mg, 89%). ¹H-NMR (600 MHz, CDCl₃) δ 13.29 (s, 1H), 7.42 (m, 4H), 7.38 (t, 2H, *J* = 7.2 Hz), 7.33 (t, 3H, *J* = 7.2 Hz), 7.26 (t, 1H, *J* = 7.2 Hz), 6.83(d, 1H, *J* = 9.0 Hz), 6.78 (dd, 2H, *J* = 6.0 and 1.8 Hz), 6.27 (s, 1H), 5.12 (s, 2H), 5.10 (s, 2H), 3.87 (s, 3H), 3.84 (s, 3H), 3.76 (s, 3H), 3.25 (t, 2H, *J* = 7.2 Hz), 2.89 (t, 2H, *J* = 7.8 Hz); ¹³C-NMR (150 MHz, CDCl₃) δ 204.8, 159.0, 155.2, 148.0, 137.2, 135.7, 134.9, 134.0, 128.7, 128.5, 128.2, 127.7, 127.3, 127.2, 120.9, 114.7, 111.9, 108.4, 97.3, 71.0, 70.5, 61.1, 61.0, 56.1, 45.0, 29.9 ; HRMS (ESI): mass calcd for C₃₂H₃₂O₇ [M + H]⁺, 529.2226; found, 529.2207.

7-(benzyloxy)-3-(3'-(benzyloxy)-4'-methoxybenzyl)-5,6-dimethoxy-4H-chromen-4-one (19)

To a solution of the dibenzylated dihydrochalcone (**18**) (93 mg, 0.2 mmol) in toluene (5 mL) was added *N,N*-dimethylformamide dimethyl acetal (43 mg, 0.36 mmol). After refluxing for 6 h, the reaction mixture was cooled and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate / *n*-hexane = 1 : 2) to afford the 4-chromone (**19**) (76 mg, 80%). ¹H-NMR (600 MHz, CDCl₃) δ 7.44 (d, 2H, *J* = 8.4 Hz), 7.40 (t, 4H, *J* = 7.2 Hz), 7.34 (t, 1H, *J* = 6.0 Hz), 7.29 (t, 2H, *J* = 7.2 Hz), 7.22 (m, 2H), 6.80 (s, 2H), 6.77 (s, 1H), 6.63 (s, 1H), 5.16 (s, 2H), 5.09 (s, 2H), 3.95 (s, 3H), 3.89 (s, 3H), 3.93 (s, 3H), 3.62 (s, 2H); ¹³C-NMR (150 MHz, CDCl₃) δ 175.9, 156.6, 154.5, 152.8, 151.0, 148.3, 148.0, 140.6, 137.1, 135.6, 131.2, 128.7, 128.4, 128.3, 127.7, 127.4, 127.2, 125.0, 121.8, 115.3, 113.1, 112.0, 97.4, 71.0, 70.8, 62.1, 61.5, 56.1, 30.8 ; HRMS (ESI): mass calcd for C₃₃H₃₀O₇ [M + H]⁺, 539.2070; found, 539.2049.

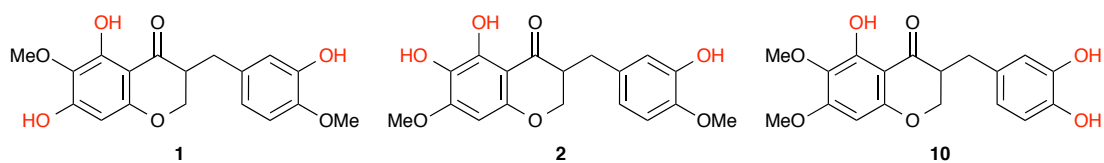
7-hydroxy-3-(3'-hydroxy-4'-methoxybenzyl)-5,6-dimethoxychroman-4-one (20)

A solution of the chromone (**19**) (35 mg, 0.07 mmol) and 10% Pd/C (10 mg) in MeOH was placed under an atmosphere of hydrogen. After stirring for 1 h, the reaction mixture was diluted with ethyl acetate, filtered through a Celite pad and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate : *n*-hexane = 1 : 1) to afford the 4-chromanone (**20**) (22 mg, 87%). ¹H-NMR (600 MHz, CD₃OD) δ 6.82 (d, 1H, *J* = 14.4 Hz), 6.67 (d, 1H,

$J = 1.8$ Hz), 6.63 (dd, 1H, $J = 8.4$ and 2.4 Hz), 6.16 (s, 1H), 4.21 (dd, 1H, $J = 11.4$ and 4.2 Hz), 4.04 (dd, 1H, $J = 11.4$ and 7.2 Hz), 3.82 (s, 3H), 3.79 (s, 3H), 3.75 (s, 3H), 3.00 (dd, 1H, $J = 13.2$ and 4.2 Hz), 2.66 (m, 1H), 2.58 (dd, 1H, $J = 13.8$ and 10.8 Hz); ^{13}C -NMR (150 MHz, CD_3OD) δ 192.4, 160.0, 158.5, 154.4, 146.3, 146.2, 136.4, 131.2, 119.9, 115.6, 111.5, 107.3, 99.1, 68.6, 60.4, 60.1, 55.0, 48.2, 32.0; HRMS (ESI): mass calcd for $\text{C}_{19}\text{H}_{20}\text{O}_7$ $[\text{M} + \text{H}]^+$, 361.1287; found, 361.1270.

Cremastranone (**1**)

To a solution of 7-hydroxy-3-(3-hydroxy-4-methoxybenzyl)-5,6-dimethoxychroman-4-one (**20**) (113 mg, 0.315 mmol) in CHCl_3 (3 mL) was added TMSI (89 μL , 0.63 mmol) at 0°C and the reaction mixture was stirred at RT for 30 min. The mixture was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (ethyl acetate / *n*-hexane = 1 : 1) to afford cremastranone (**1**) (95 mg, 87%). ^1H -NMR (600 MHz, CD_3OD) δ 6.85 (d, 1H, $J = 8.4$ Hz), 6.70 (d, 1H, $J = 1.8$ Hz), 6.68 (dd, 1H, $J = 8.4$ and 2.4 Hz), 5.91 (s, 1H), 4.23 (dd, 1H, $J = 11.4$ and 4.2 Hz), 4.06 (dd, 1H, $J = 11.4$ and 7.2 Hz), 3.82 (s, 3H), 3.77 (s, 3H), 3.08 (dd, 1H, $J = 13.8$ and 4.8 Hz), 2.82 (m, 1H), 2.63 (dd, 1H, $J = 13.8$ and 4.2 Hz); ^{13}C -NMR (150 MHz, CD_3OD) δ 200.1, 160.6, 160.1, 156.8, 147.8, 147.6, 132.2, 130.4, 121.3, 117.0, 112.9, 102.9, 95.7, 70.3, 60.9, 56.4, 47.9, 33.1; HRMS (ESI): mass calcd for $\text{C}_{18}\text{H}_{18}\text{O}_7$ $[\text{M} + \text{H}]^+$, 347.1131; found, 347.1118. Purity >95% by LC-MS.

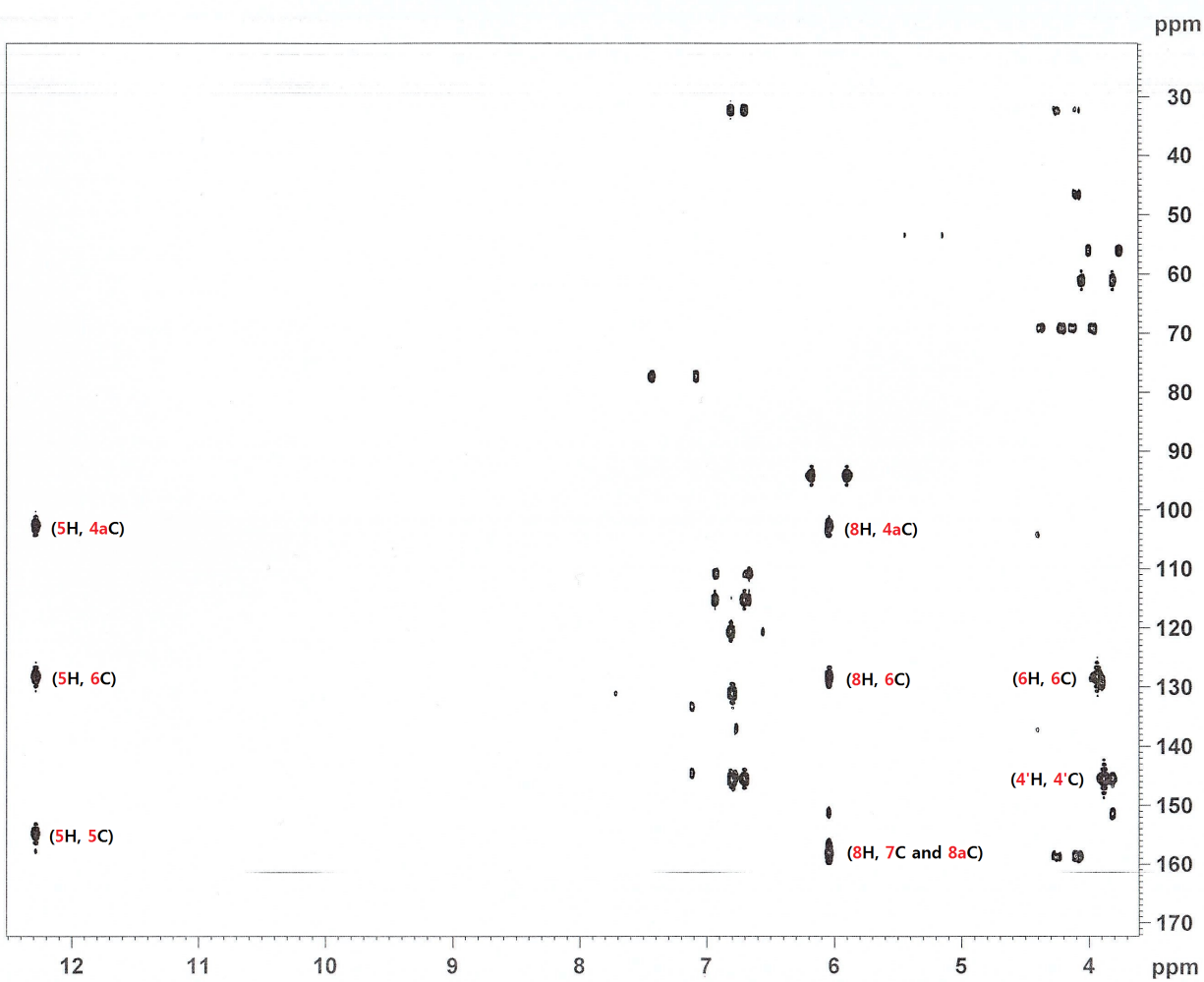
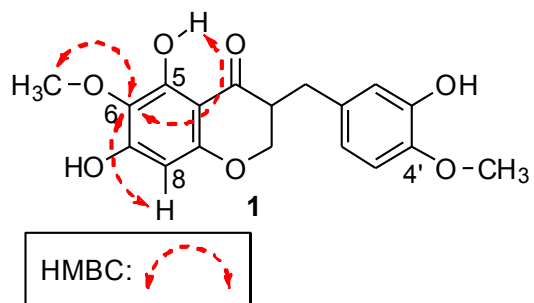


ESI Table 1. Chemical shifts in ^1H - and ^{13}C -NMR for selected compounds.

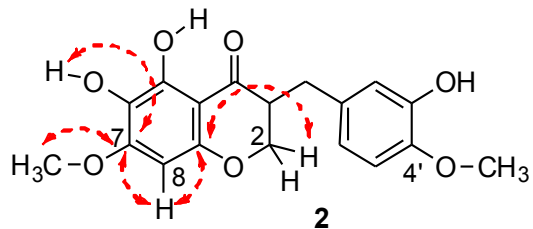
	Reported natural product (1) ²		New synthetic compound (1)		Synthetic compound (2) by TMSI		Synthetic compound (10) by BBr ₃	
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
2	4.06, 4.23	70.3	4.07, 4.23	70.3	4.11, 4.25	70.6	4.13, 4.29	70.6
4	2.81	200	2.82	200.1	2.87	200.6	2.85	200.6
4a	-	103		102.9		103.5	-	103.7
5	12.22	156.8		156.8		157.5	-	156.3
6	<u>3.82^a</u>	129.2	<u>3.82^a</u>	130.4	<u>3.87^a</u>	128.7	3.87	
7	9.87	160.9		160.7		149.3	-	162.4
8	5.91	95.8	5.91	95.8	6.14	97	6.15	92.6
8a		160.1		160.1		148.7	-	160.7
9	2.62, 3.06	33.1	2.63, 3.08	33.1	2.69, 3.12	33.2	2.62, 3.06	33.2
1'	-	132.3		132.3		132.4	-	130.8
2'	6.71	117	6.71	117	6.73	117.1	6.67	117.2
3'	8.75	147.8		147.8		147.9	-	146.6
4'	<u>3.77^a</u>	147.8	<u>3.77^a</u>	147.7	<u>3.83^a</u>	147.7	3.74	145.3
5'	6.85	112.9	6.85	112.9	6.8	112.5	6.71	116.6
6'	6.66	121.3	6.66	121.3	6.67	121.4	6.56	121.6
6-OCH₃	-	61		60.9		61.6	-	61.2
7-OCH₃	-						-	
3'-OCH₃	-						-	
4'-OCH₃	-	56.4		56.4		56.5	-	56.8

a : revised assignment by HMBC

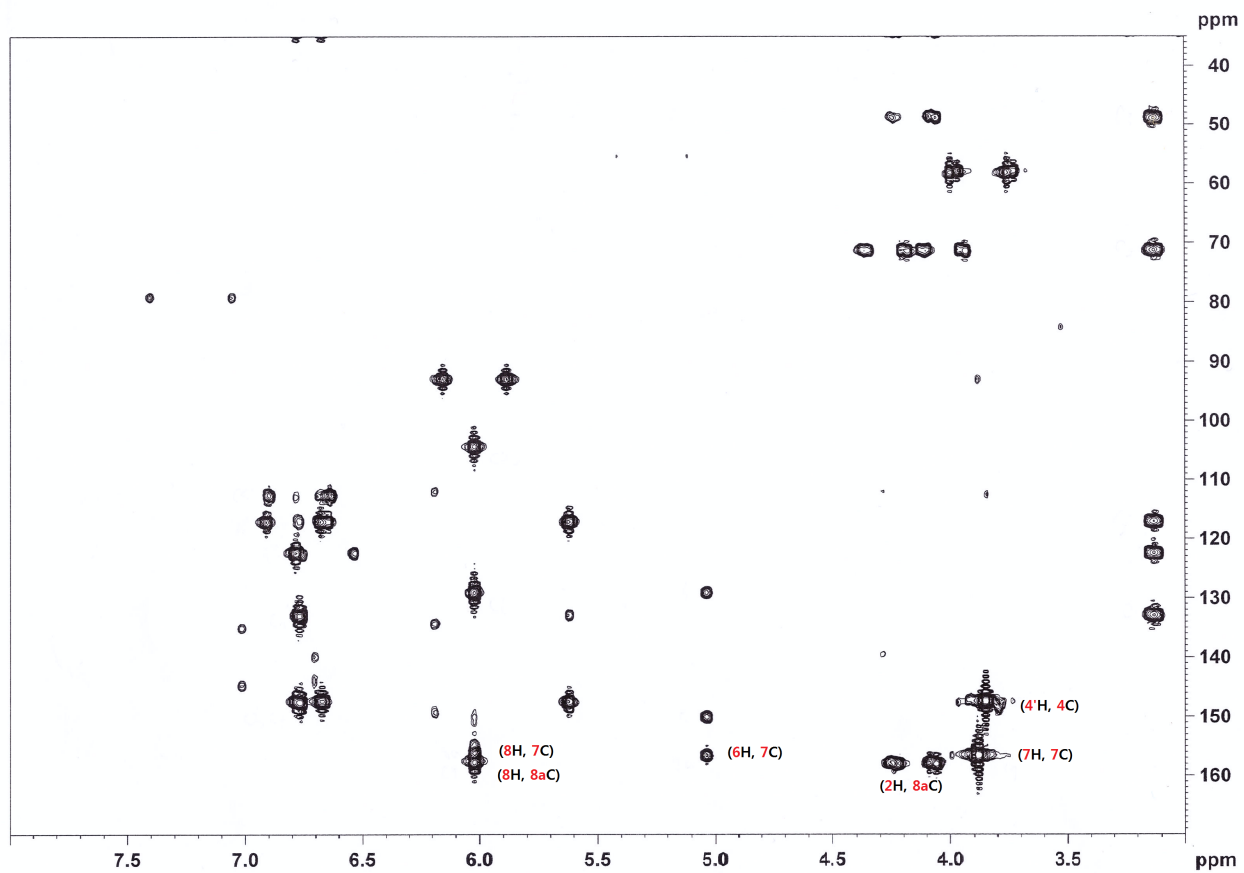
HMBC (600MHz, CD₃OD) of Cremastranone (1)



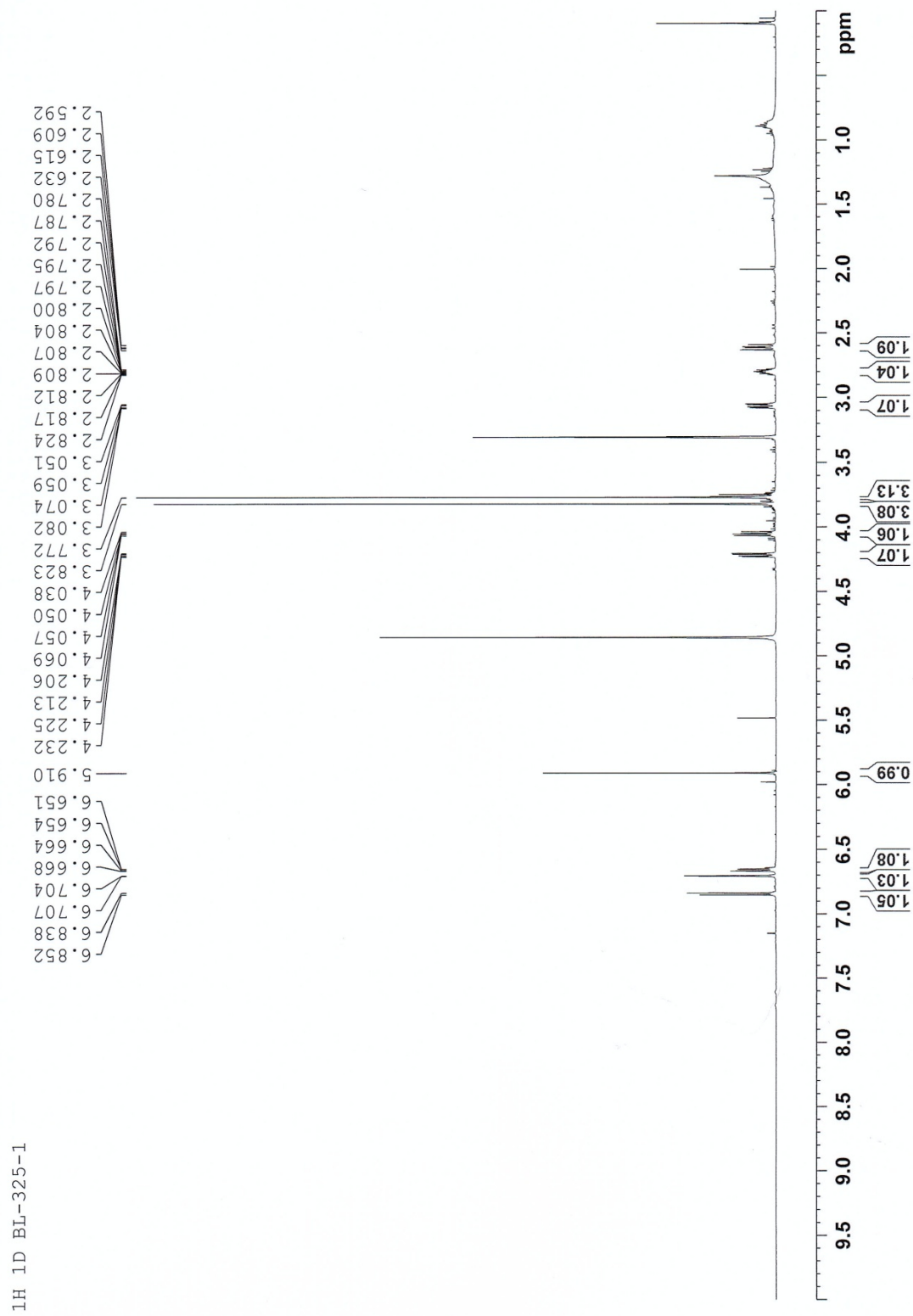
HMBC (600MHz, CDCl₃) of SH-11052 (2)



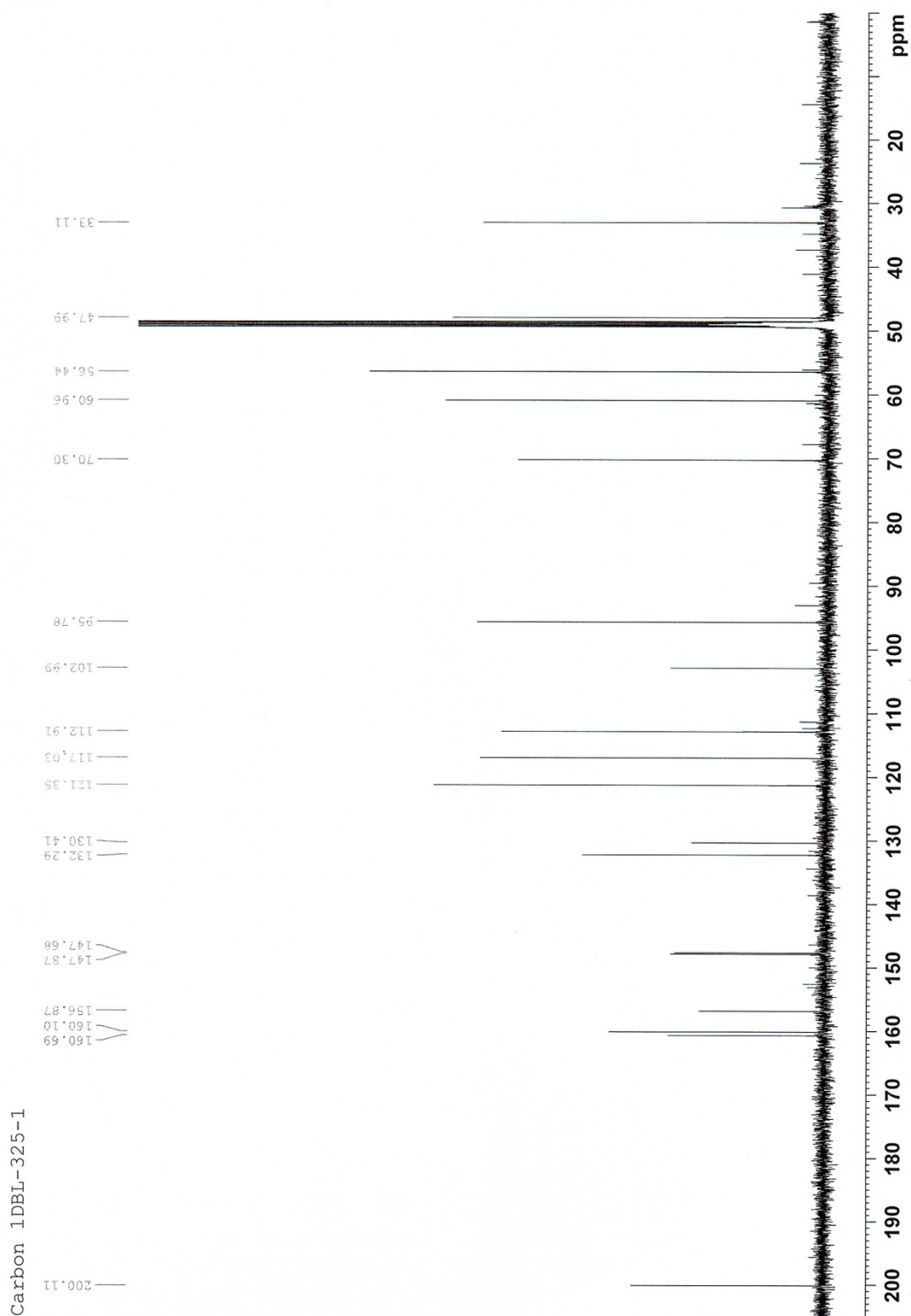
HMBC:



¹H-NMR (600MHz, CD₃OD) of Cremastranone (1)



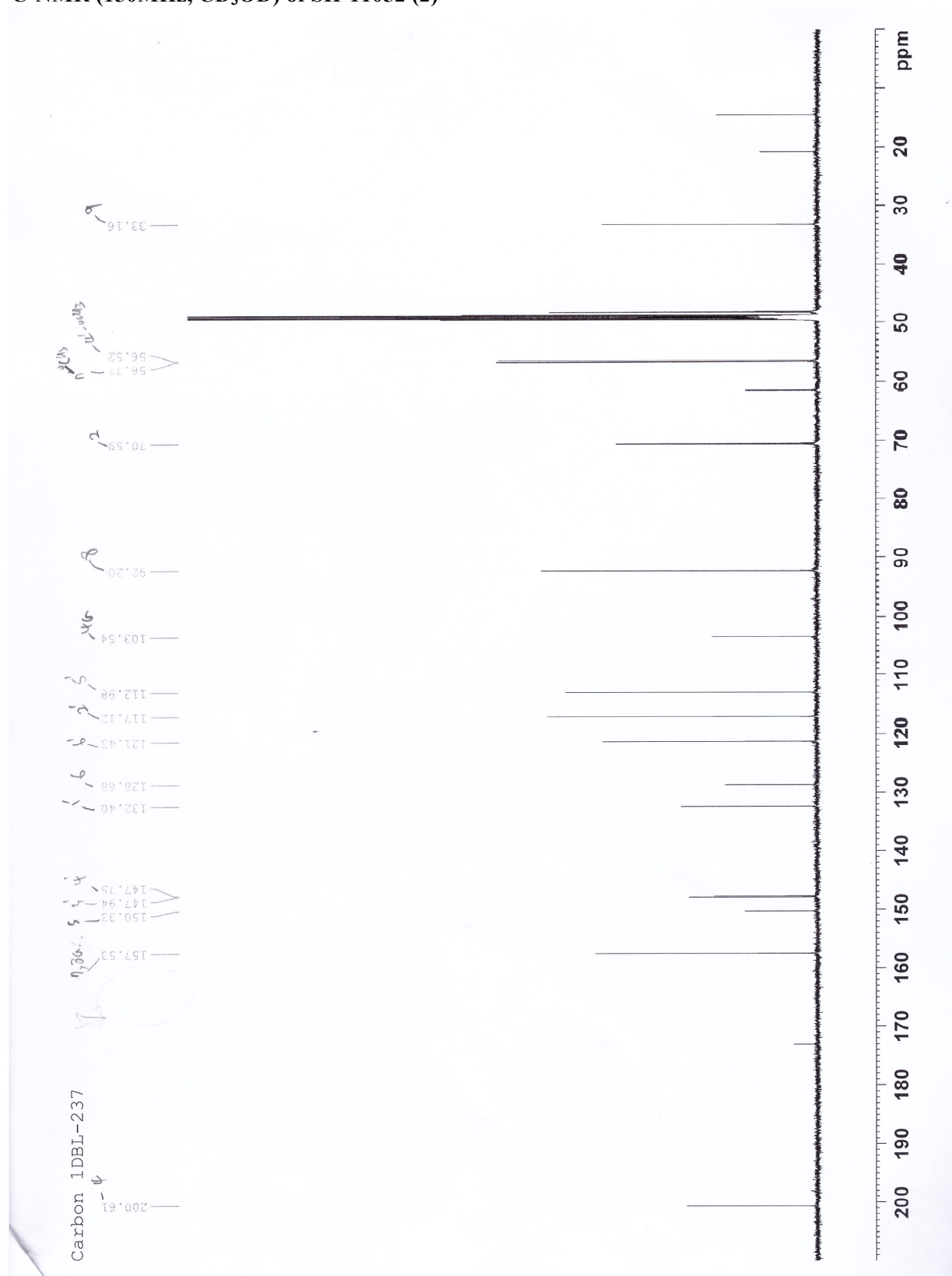
^{13}C -NMR (150MHz, CD_3OD) of Cremastranone (1)



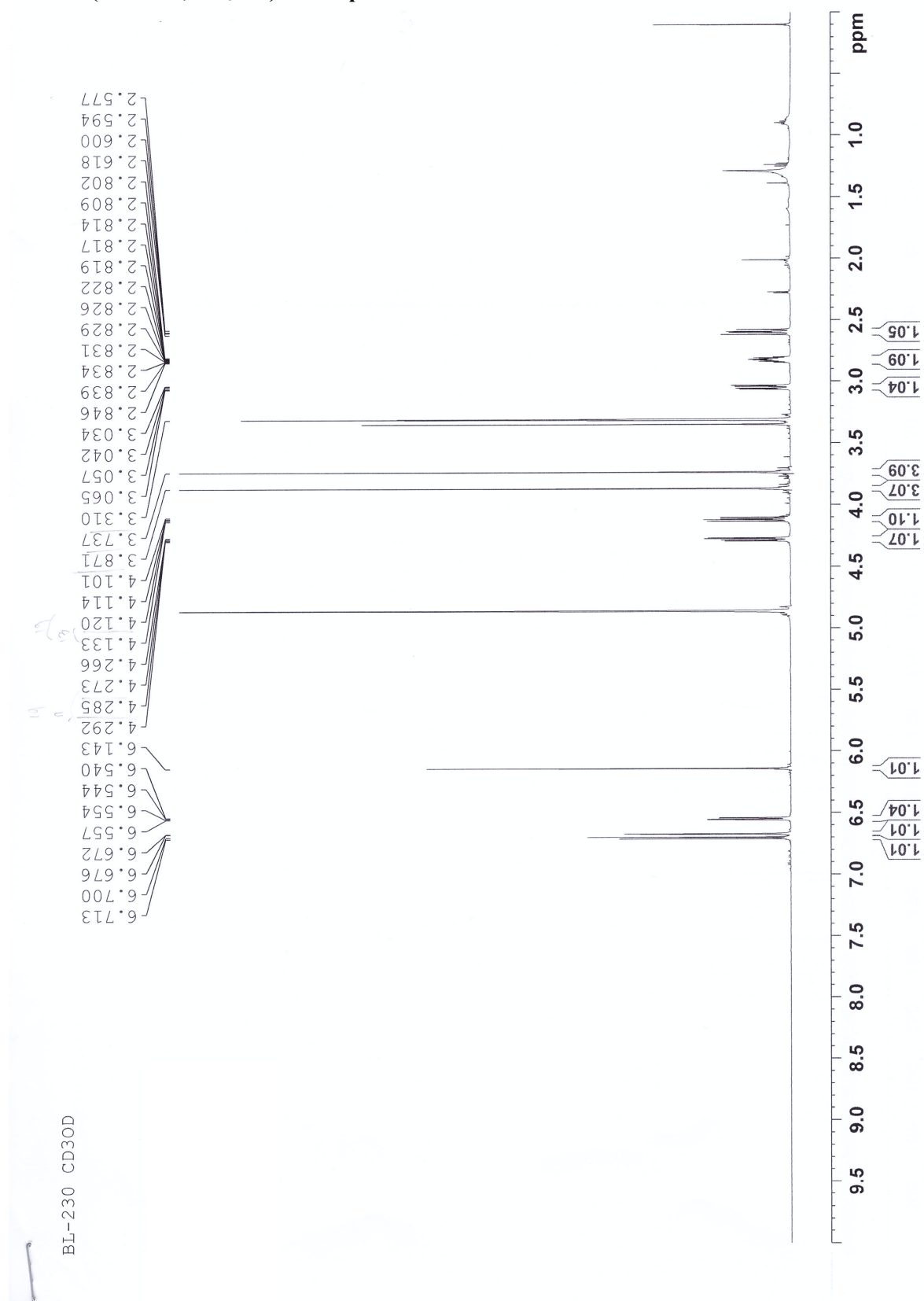
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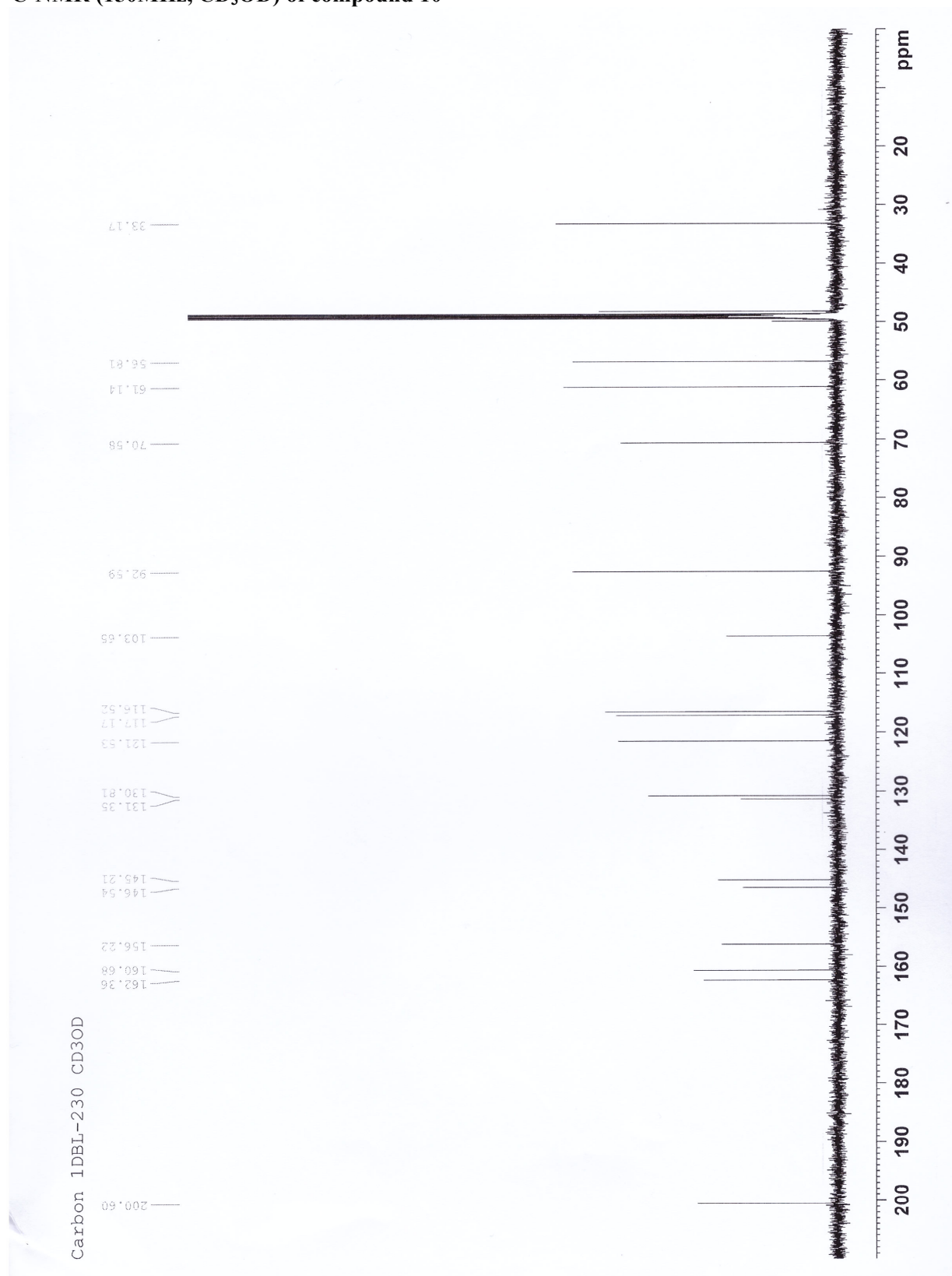
^{13}C -NMR (150MHz, CD_3OD) of SH-11052 (2)



¹H-NMR (600MHz, CD₃OD) of compound 10



^{13}C -NMR (150MHz, CD_3OD) of compound 10



Biological Methods

Cell proliferation assay

The proliferation of cells was monitored by an alamarBlue based fluorescence assay as described previously.¹ Briefly, 2500 cells in 100 μ L growth medium were incubated in 96-well clear bottom black plates at 37 °C, 5% CO₂ for 24 hours followed by 44 hours incubation with different concentrations of **1** in DMSO (range: 0.5 nM to 500 μ M; final DMSO concentration 1%). Cells used were: Human umbilical vein endothelial cells (HUVECs; Lonza) and human retinal endothelial cells (HRECs; Cell Systems) in EGM-2 medium (Lonza); Y-79 retinoblastoma cells³ (a kind gift of Dr. Brenda L. Gallie, Ontario Cancer Institute) in previously described medium;⁴ 92-1 uveal melanoma cells⁵ (a kind gift of Dr. Martine Jager, University of Leiden) in RPMI-1640, 10% FBS, penicillin/streptomycin; and ARPE-19 human retinal pigment epithelial cells⁶ (a kind gift of Dr. Michael Boulton, Indiana University) in Ham's F10, 10% FBS, penicillin/streptomycin. For VEGF-induced proliferation, HRECs plated as above were starved with EBM-2 medium (Lonza) overnight, then incubated for 44 hours with different concentrations of **1** plus 50 ng/mL human VEGF-165 (BioLegend) in EBM-2. At the end of each incubation, 11.1 μ L of alamarBlue reagent (AbDSerotec) was added and 4 hours after, fluorescent readings were taken on a Synergy H1 plate reader (Biotek) with excitation and emission wavelengths of 560 nm and 590 nm respectively. Data were analysed and dose response curves generated using GraphPad Prism software (v. 6.0).

EdU incorporation assay

The assay was carried out as described before.¹ HRECs (25,000) were seeded onto coverslips coated with attachment factor and grown for 24 hours before starving in serum-free EBM-2 medium. After starvation for 12 hours, the cells were incubated with 10 μ M 5-ethynyl-2'-deoxyuridine (EdU) in the presence of various concentrations of **1** for 8 hours. Then the cells were processed according to the manufacturer's instructions for the click-iT EdU assay kit (Life Technologies). The images were taken using an EVOS microscope (AMG) and data were analysed using ImageJ.

In vitro angiogenesis assay

Matrigel based tube formation assay was performed to monitor the tube-formation ability of HRECs in the presence of **1** as described previously.¹ Briefly, 7500 cells in 100 μ L EGM-2 medium were incubated in the presence or absence of **1** in 96-well clear plates coated with 75 μ L of Matrigel basement

membrane. After 8 hours, images were recorded using the EVOS microscope. Number of polygons was manually counted, and the tube length was measured using Angiogenesis Analyser macros⁷ in ImageJ.

In vitro scratch assay

HRECs (10⁵) were seeded in each well of a 6-well plate coated with attachment factor (Cell Systems). The cells were incubated in EGM-2 medium until confluent (~24 hours). The cells were then starved for 12 hours in serum free EBM-2 medium. After starvation, a straight scratch was introduced in the well by a sterile, fine 10 µL micropipette tip, and the well was rinsed twice using EBM-2 medium to remove unbound cells and debris. Then cells were incubated in EGM-2 medium in the presence of the indicated concentrations of **1** at 37 °C and 5% CO₂. After 8 hours, images were taken using the EVOS microscope and the number of migrated cells into the scratched area was counted.

References

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